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PATENT

METHODS AND COMPOSITIONS INCLUDING A 13kD B. BURGDORFERI PROTEIN

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INTRODUCTION

Lyme disease is a complex, multisystemic illness caused by at least three genomic species of the spirochete *B. burgdorferi sensu lato* (reviewed in ref. 6). Virtually all North American isolates have been classified as *B. burgdorferi sensu stricto* (1, 17, 63). European isolates also include two other genomic species, *B. garinii* and *B. afzelii* (1, 24). The clinical features and epidemiology of Lyme disease have been well characterized (reviewed in ref. 6). Comparatively less, however, is known about the pathogenic features of Lyme disease borrelias and immunopathological responses to them in the host.

Ignorance of precise mechanisms of Lyme disease pathogenesis is partly attributable to the paucity of basic information about all spirochetes. The spirochete cell is unique in several aspects (33). One of the features of borrelia is the abundance of one or several lipoproteins in the outer cell membrane (16, 19, 20, 34, 43). Much has been learned about immunogenicity, as well as biochemical and genetic aspects, of these lipoproteins in Lyme disease and relapsing fever borrelias (4, 16, 19, 35, 37, 42, 64).

The lipoproteins OspA and OspB are major contributors to antigenic distinctness of Lyme disease borrelias (6). Both OspA and OspB are co-transcribed from a single operon located on linear plasmid of 49 kb in *B. burgdorferi sensu stricto* (16). Many of European and some North American *B. burgdorferi sensu lato* strains express a third immunodominant major protein, OspC (64). Another protein of this group, OspD, has been also reported (43). Proteins called "OspE" and "OspF" have been reported, but their surface exposure and location in the outer membrane have not been established (39).

OspA and OspB may contribute to the spirochete's ability to adhere to or invade host cells (15, 26, 62). It has been suggested that OspA may affect the chemotactic response of human neutrophils in vitro (15). Mitogenic and cytokine-stimulatory properties of OspA and OspB have been also shown (41). In a previous study we found that reduced size and amounts of OspB was associated with lowered infectivity (48). The findings of Cadavid *et al* indicated that differences in invasive properties and tissues tropism between serotypes of related spirochete *Borrelia turicatae*, a relapsing fever agent, may be determined by the

expression of a single surface protein that is analogous to Osp proteins of *B. burgdorferi* (23).

These studies of function of Osp proteins, however, are still limited in number. More information is needed regarding the function of these proteins, in particular their roles in infectivity and their contributions to the microorganism's ability to survive in the host. One approach to obtain these insights is selection and characterization of mutants with altered surface lipoproteins. There were several compelling reasons for studying *B. burgdorferi* cells that lacked all known Osp proteins (51, 54). Our first intent was to characterize morphology and function of the Osp-less mutant. We asked whether borrelias lacking OspA, B, C, and D would be altered in such functional properties, as (i) generation time, (ii) ability to form colonies on solid medium, (iii) adherence to cells, (iv) serum and complement sensitivity, (v) potential to evoke immune response after intradermal live cell inoculation, and (vi) ability to survive in the skin. Among pathogenic borrelias the role of surface lipoproteins in these respects have not yet been reported.

Another intriguing aspect was the immunological characterization of the Osp-less mutant. There have been several studies describing low molecular weight lipoproteins that have not been identified as Osps. Katona *et al* showed the presence of a major low-molecular-weight lipoprotein specific for *B. burgdorferi* and raised the possibility that it was a borrelial equivalent of Braun's lipoprotein (36). Another study reported an immunogenic 14 kDa surface protein of B. burgdorferi recognized by sera from Lyme disease patients (55). These findings encouraged us to determine whether other proteins are present on the surface in the absence of Osp proteins.

SUMMARY OF INVENTION AND DESCRIPTION OF PREFERRED EMBODIMENTS

MATERIALS and METHODS

Strains and culture conditions

B. burgdorferi sensu stricto mutants were of the B31 (ATCC 35210) lineage (Table 1). The Osp phenotypes and plasmid contents of noninfectious derivatives B311, B312, B313 and B314 were described previously under these or other designations (3, 7, 32, 54).

Table 1. Isolates of *B. burgdorferi sensu lato* used in the study and their Osp profile

Genomic species	Isolate	Osp profile[a]				Reference
		OspA	OspB	OspC	OspD	
B. burgdorferi	B31	+	+	-	+	22, 54
	B311	+	+	-	-	3, 7, 54
	B312	+	+	+	-	32, 54
	B313	-	-	-	-	51, 54
	B314	-	-	+	-	54
	HB19	+	+	+	+	12, 60
	Sh.2	+	+	+	-	57
B. afzelii	ACAI	+	+	+	-	17
B. garinii	IP90	+	+	+	-	1, 17

[a] Osp profile was determined by Western blot analysis.

Populations that were passed in medium not more than 10 times were considered low passage isolates. The low passage, infectious progenitor for this lineage retained the original strain designation, B31 (22). With the exception of B31, all cells of this lineag were grown from single cell clones. In some experiments we also used these other strains (Tabl 1): HB19 (12, 60) and Sh.2 (57), both of which are *B. burgdorferi sensu stricto*, *B. afzelii* strain ACAI (17) and *B. garinii* strain Ip90 (1, 17). *B. hermsii* HS1 serotype 33 (ATCC 35209; ref. 11) was abbreviated to Bh33. Borrelias were grown in BSK II medium and harvested by methods described previously (3, 5). When culturing tissues from animals, rifampicin (50 µg/ml), phosphomycin (100 µg/ml) and, for skin samples, additionally amphotericin (25 µg/ml) were added to the medium. Cells were counted in a Petroff-Hauser chamber by phase-contrast microscopy. In some experiments borrelias were also grown on solid BSK II medium as described (32, 51). To estimate growth rate, borrelias at an initial concentration of 2×10^6 cells/ml, were grown in tightly capped, 13 x 100-mm polystyrene culture tubes (Falcon Labware, Lincoln Park, NJ) containing 6 ml of medium. Growth at 34°C in 1% CO_2 atmosphere was monitored visually and by cell counts every 12 h for 3 d. The amount of total cellular protein in the final cell pellet was determined with Bradford reagent (Bio-Rad Laboratories, Richmond, CA, (12). The microscopic aggregation of borrelias alone or in the presence of antibodies was graded according to the following scale: 0, single cells with less than 10% of the cells in clumps of 2-10 cells; 1+, 10-50% of cells in clumps of 2-10; 2+, 10-50% of cells in clumps of 11-100; 3+, >50% of cells in clumps of 11-100; and 4+, >50% of cells in clumps of ≥100.

Antisera and monoclonal antibodies

The origins of the OspA-specific mAb H5332 (12), OspB-specific mAb H6831 (10) and Vmp33-specific mAb H4825 (10) have been given. Monoclonal antibody H9724 binds to native and denatured flagellins of different Borrelia species (9). These antibodies are IgG subclass 2a (IgG2a).

Additional polyclonal and monoclonal antibodies were produced for this study. Female, 6-8 week old BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used.

Freshly-harvested borrelia were washed with and resuspended in PBS, pH 7.0. The total cellular protein in the suspension was estimated with Bradford reagent and adjusted with PBS for a total protein concentration of 200 µg/ml. 0.5 ml of antigen suspension was emulsified in 0.5 ml of complete Freund's adjuvant (CFA; Sigma), and 200 µl of emulsion was administered as six subcutaneous injections at day 0. Control mice received a 200 µl emulsion of equal parts of CFA and PBS alone. The total dose per mouse was 20 µg protein. After 4 weeks mice were boosted with the same dose. Mice were bled by eye sinus puncture 10 days after the boost. After collection, sera were evaluated by ELISA and GIA. On day 52, the mice received intravenously 2×10^8 viable borrelias in 100 µl of PBS. Fusion of mouse splenocytes with NS1 myeloma cells were performed on day 56 by a modification of the method of Oi and Herzenberg (44). Undiluted hybridoma supernatant fluids without antibiotics were screened by wet ELISA, unfixed cell IFA and Western Blot techniques. Those fluids that were positive by either one of these methods were then evaluated by GIA. For GIA hybridoma supernatant fluids were dialyzed against PBS, pH 7.0 and concentrated with Centriprep-10 (Amicon) cartridges. The isotypes of antibodes were determined using a commercial kit (Immunotype; Sigma). Ascitic fluids from hybridomas were produced as described (51).

Purified mAbs and univalent Fab fragments were prepared from hybridoma supernatants essentially as described (53). Briefly, hybridoma supernatants were concentrated using an Amicon 8200 membrane concentrator with a Diaflo YM30 ultrafiltration membrane (Amicon, Beverly, MA) under 50 psi N_2. Purified mAbs were obtained by protein A-sepharose column chromatography. Univalent Fab fragments were prepared using the Immunopure Fab Preparation kit (Pierce) by cleaving the purified antibodies with papain, retaining intact immunoglobulin and Fc fragments on a protein A-sepharose column, and dialyzing the void volume of the column against PBS, pH 7.0. Purifi d mAbs and Fab fragments were concentrated with Centriprep-10 (Amicon). Protein concentrations were determined by UV spectrophotometry at 280 nm. Purified whole IgG and Fab fragments were analyzed by SDS-PAGE. Reactivities of purified mAbs and Fab

fragments were confirmed by direct and indirect immunofluorescence assay, Western blot and GIA.

ELISA

The method for ELISA was essentially as described previously (52). For this "dry" ELISA borrelias at a total protein concentration of 1.4 µg/ml in phosphate-buffered saline (PBS), pH 7.0 were dried onto polystyrene 96-well microtiter plates at 37°C for 18 h. For a "wet" ELISA borrelias at a total protein concentration of 3 µg/ml in 15 mM Na_2CO_3-35 mM $NaHCO_3$ buffer, pH 9.6 were coated onto plates at 4°C for 24 h. After blocking for 1 h at 37°C with 1% (wt/vol) dried nonfat milk in PBS (milk/PBS) and washing with PBS alone, twofold dilutions of antibody in milk/PBS were added. The plates were incubated for 2 h at 37°C and washed with PBS. Bound antibody was measured using alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed). The substrate was *p*-nitrophenyl phosphate (Sigma). Absorbance values were recorded at 490 nm on a model 580 ELISA reader (Dynatech Laboratories, Chantily, VA); wells with values $\geq$ 0.2 were considered positive.

Immunofluorescence assays

Indirect immunofluorescence assay (IFA) of fixed, dried cells was performed as described (11, 12). Harvested, fresh borrelias were washed with RPMI 1640 medium, mixed with a suspension of washed rat erythrocytes in 50% RPMI 1640-50% fetal calf serum, and a thin smear of the suspenion was coated on the slides. Slides were fixed in methanol, air dried, and kept in a dessicator at -20°C until use.

Binding of monoclonal antibodies (mAb) to unfixed live spirochetes was assessed by a modification of the procedure of Barbour *et al* (12). 10^7 borrelias were washed with 2% (wt/vol) BSA in PBS/Mg (PBS/Mg/BSA) and then resuspended in 0.5 ml of undiluted hybridoma culture supernatant or 0.5 ml of PBS/Mg/BSA containing the mAb of interest. The cell mixture was incubated at room temperature with gentle rotation for 60 min. The cells were centrifuged, washed twice with PBS/Mg/ BSA, resuspended in 30 µl volume of PBS/Mg/BSA with 20 µg/ml of anti-mouse Ig-fluorescein $F(ab')_2$ fragment (Boehringer-Mannheim, Indianapolis, IN) and incubated for 30 min under the same conditions. Before

microscopic evaluation the volume of the cell suspension was adjusted to 300 µl with PBS/Mg/ BSA.

For direct IFA purified mAbs and their Fab fragments were conjugated with fluorescein isothiocyanate (QuickTag FITC Conjugation Kit; Boehringer-Mannheim). Fractions containing the antibody-fluorescein conjugate were mixed together, dialyzed in the dark against PBS for 24 h, and concentrated with a Centriprep-10 (Amicon, Beverly, Mass.). 10^7 borrelias in log-phase growth were resuspended in RPMI 1640 medium with 10-100 µg/ml of antibody-fluorescein conjugate and examined for fluorescence at 3, 15, 30, 60, and 360 min.

Growth inhibition assays

The growth inhibition assay (GIA) was described previously (53). Briefly, to a 100 µl volume of BSK II containing 2 x 10^6 borrelias was added an equal volume of heat-inactivated (56°C for 30 min) mAb or polyclonal antiserum, serially diluted two-fold in BSK II. To evaluate the susceptibility of borrelias to fresh, nonimmune serum, we applied the same growth inhibition technique using pooled unheated serum from C3H/HeN mice (Taconic, Germantown, N.Y.). Blood was drawn on ice, separated from red blood cell clot, and immediatelly frozen at -135°C. Heat-inactivated serum from the same mice served as a control. To determine the susceptibility of borrelias to complement, unheated or heated (56°C for 30 min) guinea pig complement (Diamedix, Miami, FL) was added to each well at an activity ranging from 6 to 1 hemolytic unit (HU; CH_{50}) per well. In some experiments, 2 HU of unheated guinea pig complement were added to each well for a final concentration of 10 HU/ml of medium after addition of antibody. The incubations were performed in flat-bottomed, 96-well, polystyrene microtiter plates, covered by adhesive, clear plastic seals (Sensititre Microbiologic Systems, Westlake, OH) and were carried out for 72 h at 34°C in a 1% CO_2 atmosphere. Growth in the wells was monitored visually for changes in the color of the phenol red indicator and by phase contrast microscopy of wet-mounts of culture samples. A pink color of the indicator after incubation represented at least 20-fold fewer cells in these wells than in wells that were yellow. The minimal inhibitory concentration

(MIC) was the lowest concentration of mAb that produced pink instead of yellow wells (53). All growth inhibition studies were performed at least twice.

Electrophoresis and Western Blot analysis

Whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% or 17% acrylamide as described previously (2, 11). In some experiments, cleavage of surface-exposed proteins of intact borrelias with proteinase K (Boehringer-Mannheim) was carried out (51). For this study 490 µl of a suspension containing 5×10^8 cells in PBS/Mg was mixed with 10 µl of proteinase K solution (20 mg/ml of water) and incubated for 40 min at 22°C. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride. For Western blot analysis, proteins were transferred to nitrocellulose membranes, which were then blocked with 3% (wt/vol) dried nonfat milk in 10 mM Tris-HCl (pH 7.4)-150 mM NaCl (milk/TS) for 2 h as desribed before (44). After a wash in milk/TS, membranes were incubated with mAb ascitic fluid diluted 1:50 or 1:100 in milk/TS or hybridoma supernatant fluid diluted 1:5 or 1:10 in milk/TS. Alkaline phosphatase-conjugated recombinant protein A/G (Immunopure; Pierce Chemical Co., Rockford, IL) served as the second ligand. The blots were developed with nitro-blue tetrazolium chloride-5-bromo-4-chloro-3-indolylphosphatase *p*-toluidine salt (Pierce, Rockford, IL).

Adherence assay

An assay for adherence of intrinsically-labeled borrelias to human umbilical vein endothelium (HUVE) cells was carried out essentially as described (62). Briefly, borrelias were intrinsically radiolabeled with [^{35}S]-methionine, washed with PBS and resuspended to a density of 1.7×10^8 cells per ml in Medium 199 with 20% fetal calf serum. 300 µl aliquots of radiolabeled spirochetes were added to confluent HUVE cell monolayers grown in 24-well plates. After a 4 hr incubation at 4°C, monolayers with associated organisms were washed, solubilized, mixed with scintillation cocktail (Universol ES; ICN Pharmaceuticals, Irvine, CA), and counted by scintillation. The assay was done with triplicate samples and performed twice. Differences between borrelia populations in adhesion were analyzed by

Student's t test.

Experiments in mice

Six-to-eight week old, female C3H/HeN mice (Taconic, Germantown, N.Y.) were used. Borrelia cells were counted and diluted in BSK II to give the desired inoculum. For live cell immunization, 100 µl of cells in BSK II medium was transferred to 900 µl of sterile PBS solution immediately prior to immunization. 100 µl of this suspension then was inoculated intradermally in the abdomen at day 0. As a control, 100 µl of 0.1X BSK II in PBS was used. On day 24 mice were bled from the tail vein, and their sera were examined by ELISA and GIA. Mice were challenged on day 28 at the base of the tail with 10^4 of *B. burgdorferi* strain Sh.2 (48). Mice were euthanized 14 d following infection. Plasma (0.5 ml) obtained from citrated blood, the whole bladder, macerated heart, and cross-cuttings of both tibiotarsal joints were added to BSK II medium and cultured at 34°C. Cultures were examined for the presence of motile spirochetes by phase-contrast microscopy at days 7 and 14 of cultivation; they were scored as negative when no motile spirochetes were seen in forty 400X fields. For evaluation of borrelia survival in skin, borrelias were diluted in 1X BSK II. The abdominal skin was shaved, and 10^7 borrelia cells were injected intradermally at 3 or 4 separate locations. Mice were sacrificed at 0.25, 0.5, 2, 6, 9 , 12, 18 and 24 h after injection and samples of skin from the injection sites were immediately cultured in BSK II medium at 34°C.

RESULTS

Isolate B313 of the B31 lineage of *B. burgdorferi* lacked OspA, B, C, D (Table 1; 51, 54). This mutant was selected from a clonal population of B311 under the selective pressure of an anti-OspA mAb. Isolate B311's Osp profile was $OspA^+B^+C^-D^-$ (54). Mutants that lack both OspA and OspB were selected with polyclonal or monoclonal antibodies directed against *B. burgdorferi* at a frequency of 10^{-6}-10^{-5} (51). The genetic basis for the Osp-less phenotype was loss of a 38 kb and 49 kb linear plasmids and

retention of a 16 kb plasmid (43, 51, 54).

Growth rate

Osp-less mutant B313 was easily distinguishable from B311, as well as from other high-passage, Osp-bearing isolates of the B31 lineage, in broth culture by its tendency to form microscopic aggregates. B313 cultures had aggregation scores of 1+ or 2+, whereas B311 had a score of 0. Another observed difference was the decreased ability of B313 cells to turn the phenol red indicator yellow in the BSK II, even when the culture reached stationary phase. One possible explanation for this is that metabolic activity of the Osp-less mutant was lower than that of wild-type borrelias. Alternatively, the OspA$^-$OspB$^-$ mutant may have a slower rate of growth than its parent B311 and, consequently, does not reach the same cell densities as wild-type borrelias at a particular time point. To examine these possibilities we determined the growth rates of B311 and B313 and measured the amount of borrelia protein in the final cell pellet.

B311 and B313 cells were grown until stationary phase, that is, when no further growth occurred, was reached. Cell counts were determined every 12 h in triplicate, and the $\log_{10}$ of means of cell counts were plotted against time. At stationary phase B311 cultures had a cell density of 1.5-2.0 x 10^8 cells/ml and B313 cultures had a cell density of 4-5 x 10^7, fourfold lower. Protein concentrations in the final B311 and B313 cell pellets were 0.65 mg and 0.16 mg, respectively, a finding consistent with the cell counts. The mean generation time (± standard error of the mean) of B311 cells was 6.6 ± 0.1 h; the values for B313 cells were 9.5 ± 0.2 h, 50% slower. These findings indicated that the Osp-less cells both grew more slowly and achieved a lower final cell mass than did their Osp-bearing counterparts.

Plating efficiency

We also evaluated another biological characteristic of the Osp-less mutant, namely, its ability to grow as a colony on solid medium. Current procedures for cultivation of different low and high passage *B. burgdorferi* on solid medium yield effciencies of plating between 50 and 100% (32, 48, 51). In the previous studies we found we could plate other

antibody-resistant variant populations of the B31 lineage with the same high efficiency (51). An xception was the very low plating efficiency of mutant B314 (Table 1), which lacks all lin ar plasmids and has an OspA$^-$B$^-$C$^+$D$^-$ phenotype (54). These data suggested that mutants with Osp$^-$ phenotype might also have a lesser ability to form colonies.

The experiment was performed twice, each time plating in triplicate 10^1-10^6 borrelias per plate. B311 cells grew as colonies with the expected plating efficiency of 50%. The efficiency of B313 plating was 0.01%, more than a thousand-fold lower than for B311 cells under the same conditions. Of three arbitrarily chosen colonies of B311 that grew in broth medium and were then subjected to SDS-PAGE, all retained the Osp-less phenotype (data not shown).

Adherence to endothelial cells

Adherence of radiolabeled *B. burgdorferi* B311 and B313 cells to HUVE cell monolayers was measured after 4 h at 4°C. At this temperature borrelias do not detectably enter endothelial cells and ladherence of cells becomes maximal by 4 h (27). The assay was repeated twice. Results of the two experiments are given in Table 2. The ability of Osp-less cells to adhere HUVE monolayer both times was only half that of wild-type borrelias, a difference that was significant ($P < 0.001$).

Serum and complement sensitivity

Wild-type *B. burgdorferi* is resistant to the nonspecific bactericidal activity of nonimmune serum, in spite of classical and alternative complement pathway activation (38). We asked whether the borrelias' ability to resist the nonspecific bactericidal effects of complement might be attributable to Osp proteins. Accordingly, we first exposed B311 cells and the Osp-less mutant to two-fold serially diluted fresh, naive mouse serum in a GIA. Heat-inactivated serum was applied in the same assay in parallel. As expected, B311 cells were resistant to the nonimmune serum; no growth inhibitory effect on the cells was observed at the lowest serum dilution of 1:8. In contrast, the minimum inhibitory titer of nonimmune serum against Osp-less borrelias was 1:64. In wells with inhibited growth the B313 cells were nonmotile and had large membrane blebs (data not shown). When heat-

Table 2. Adherence to HUVE cells by B311 and B313

Experiment[a]	Cell population	Adherence[b]	
		Mean cpm adhered ± SEM[c]	Mean % of inoculum adhered[d]
I	B311	14719 ± 134	5.0
	B313	7360 ± 36	2.5
II	B311	13447± 92	5.7
	B313	6801 ± 83	2.9

[a] The specific activities of inocula for adherence assays in experiments I and II were 2.9×10^5 and 2.3×10^5 cpm, respectively.

[b] Measured following incubation for 4 h at 4°C.

[c] Radioactivity bound to host cells following incubation and washing, expressed as the mean of three samples.

[d] Differences between borrelia populations in adhesion were analyzed by a Student's t test ($P < 0.001$).

inactivated serum was applied to either B311 or B313 cells, growth inhibition or these morphologic effects were not observed at a serum dilution 1:8. The findings of this experiment suggested that complement affected the Osp-less cells.

To further evaluate the serum-susceptibility of the Osp-less mutant, we next compared the effect of different activities of guinea pig complement on B311 and B313 cells. The dose of applied complement varied between 1-6 HU per well, and, as a control, the same doses of heat-inactivated complement were used. The study was performed twice. Whereas heat-inactivated guinea pig complement had no growth inhibitory effect on either isolate at the doses of 6 HU or less per well, there were substantial differences in the effect of unheated complement on B313 and B311. As little as 1 HU of complement inhibited growth of B313; this represented an MIC of ≤5 HU/ml. The corresponding MIC of unheated complement for B311 cells was ≥25 HU/ml.

We also estimated the frequency of B313 cells surviving in the presence of complement. Because of B313's poor growth on solid medium, the experiment was performed in 96-well microtiter plates (51). 5×10^6 of B311 or B313 cells were exposed to 3 HU/tube of guinea pig complement for 6 h. After this time cell suspensions were diluted to the concentration of complement less than 1 HU/tube and aliquoted in 200 µl volumes to individual microtiter plate wells at inocula ranging between 10^0-10^5 cells per well. Cells that were exposed to heat-inactivated complement or no complement at all served as controls. The frequency of complement-resistant mutants of B313 was calculated using tables of the Poisson distribution to be $3\text{-}6 \times 10^{-5}$.

Of 11 complement-resistant B313 clones that were transferred to medium without complement, only 6 proliferated. When these 6 cultures were again exposed to 3 HU of complement, all were as susceptible as the parent population. This suggested that if some changes had occurred in the cell, they most likely represented a phenotypic change. When the 6 cultures derived from the resistant populations were examined by PAGE, there was no discernable difference between them and the control B313 protein profiles (data not shown).

Survival of borrelias in skin

In the previous experiment we showed that outer surface lipoproteins might have a rol in protecting borrelias from one nonspecific host defense, namely, complement. Borrelias invade the host through the skin, being able to survive in it from a few days to years (59). Accordingly, we next evaluated whether Osp proteins also might protect borrelias from nonspecific resistance factors in the skin of the mouse, for example, different chemical substances from tissues with antibacterial activity, early inflammation factors, and phagocytic cells (18).

In a first step assessing these factors, we determined how long B311 and B313 cells would survive in the skin after intradermal inoculation. The experiment was repeated twice. In total 8 to12 separate skin locations were evaluated for spirochetal growth at the each time point. Mice were sacrificed at 0.25, 0.5, 2, 6, 9, 12, 18 and 24 h following inoculation, and full-depth skin biopsies were cultured. All cultures from up to 9 h were positive with both B311 and B313. In 12 h, 4 out of 8 and 5 out of 8 skin cultures were positive with B311 and B313 cells, respectively. None of the cultures from 18 and 24 h after inoculation was positive. These findings indicated that OspA and/or OspB might not benefit the borrelia's survival in the skin. To confirm that cells that survived in the skin retained the same phenotype, 6 randomly chosen cultures each of B311 and B313 were subjected to SDS-PAGE; all of the examined cells retained an unchanged protein profile (data not shown).

Immunization by intradermal inoculation with live cells

The next question addressed was whether live cells lacking known Osp lipoproteins were able to induce immune response in the skin and, if so, how that response differed from the one induced by Osp-bearing cells. A rationale for this epxeriment was another study, in which viable but noninfectious *B. burgdorferi* of strain HB19 at single intradermal dos of 10^6 live cells per mouse were sufficient protect mice against challenge with 10^4 Sh.2 cells 4 weeks later (49). This immunization dose was used with B311 and B313 cells in the present study. The immune responses of immunized and control mice were

valuated by ELISA and GIA with B311 and against the challenge strain, Sh.2.

As shown in Table 3 only immunization with cells expressing OspA and OspB, that is, B311, was effectiv in protecting all 5 mice from experimental infection with 10^4 cells of the challenge strain. Osp-less B313 failed to elicit a protective immune response at a immunization dose of 10^6 cells. All 5 mice that were immunized with live Osp-less mutant cells, as well as control mice injected with 0.1X BSK alone, became infected. Immune responses among the groups as evaluated by ELISA and GIA also differed substantially. Whereas B311 cells evoked an immune response as assessed by ELISA and especially by GIA, the response to B313 cells in the same assays was similar to that of the control group. Western blot analysis with sera from mice immunized with B313 showed no response to proteins of *B. burgdorferi*, except for faint bands against flagellin (52, not shown). Inasmuch as both B313 and B311 appear to survive in the skin for the same time span, a possible explanation for these results of immunization was that Osp proteins are an important stimulus for the host immune system to recognize the spirochete.

Polyclonal antisera to B311 and B313

The lack of an antibody response to B311 and other Osp-bearing cells by mice immunized with B313 might also be explained by the presence of unique antigens in B313 cells. According to this hypothesis, antibodies were produced in response to live cell immunization with B313 but they were directed against antigens found only in B313 cells. Ther have been reports indicating that *B. burgdorferi* has other surface proteinaceous antigens than those been defined as Osps (19, 36, 40, 55, 58). These considerations encouraged us to investigate the possibility of non-Osp antigens that might still be present on the surface of the mutant cells.

The previous experiment showed that there was little detectable antibody response after live cell intradermal immunization with Osp-less cells at a dose that evokes antibodies in animals immunized with Osp-bearing cells. Consequently, to study the immunogenicity of Osp-less cells another immunization approach was needed. Mice were immunized with B311 and B313 whole cell emulsified in an adjuvant and boosted once with the same

Table 3. Intradermal immunization and protection of mice with live B311 and B313

Immunogen[a]	Mouse No	ELISA[b]	GIA[c]	Experimental infection[d]
B311	1	256	1024	0/5
	2	128	128	
	3	256	512	
	4	256	512	
	5	128	128	
B313	1	4	<16	5/5
	2	8	<16	
	3	4	<16	
	4	4	<16	
	5	8	<16	
Control[e]	1	4	<16	5/5
	2	2	<16	
	3	4	<16	
	4	4	<16	
	5	4	<16	

[a] 10^6 cells were injected intradermally in the abdominal region of each mouse at day 0.

[b] Reciprocal ELISA titers of individual mouse sera against Sh.2 cells at day 24.

[c] Reciprocal growth inhibition titers of individual mouse serum with 2 HU of guinea pig complement against Sh.2 cells at the day 24.

[d] Syringe challenge with 10^4 *B. burgdorferi* strain Sh.2 was performed at the day 28 (number of mice infected/total tested).

[e] Control mice were injected with solution of 0.1X BSK II in PBS.

preparation. Sera were examined against both immunogens 7 wk after the initial immunization; the results are presented in Table 4. We first examined the immune response by dry ELISA and found that reciprocal titers for a homologous reaction were as high as 32,768. When heterologous sera were evaluated, the reciprocal titers were still high: 16,384 for anti-B311 serum against B313 cells, and 4,096 for anti-B313 serum against B311 cells. Sera from mice immunized with CFA alone were negative at a dilution of 1:2. This experiment confirmed that, besides known Osps, there were other immunogenic components recognized by mice.

Antisera pooled from within the same group were also evaluated by GIA for functional activity (Table 4). To avoid the deleterious effect of complement on Osp-less cells the serum was heat-inactivated. As expected, the reciprocal growth inhibitory titer of anti-B311 against B311 was high at 8,192. Anti-B313 serum did not effect B311 cells at any of the dilutions examined. Moreover, Osp-less mutant cells were inhibited by anti-B311 polyclonal serum only at a dilution of 1:32. The latter result, while indicating the specificity of the response, nevertheless, suggested that growth inhibitory antibodies to non-Osp components were produced. This was confirmed by examining the Osp-less mutant cells with homologous anti-B313 serum; the reciprocal growth inhibitory titer was 4,096. There was no growth inhibition of either B311 or B313 cells by sera of mice immunized with adjuvant and PBS alone.

mAbs against the Osp-less mutant

To further characterize the surface antigens of the Osp-less mutant we produced mAbs to B313. Procedures used for production and screening of hybridoma supernatant fluids were designed to select for and identify those mAbs that were directed against surface proteins and had functional activity by GIA. To enhance selection of antibodies against surface components mice were boosted intravenously with live B313 before the spleen fusion. As a screen for surface-directed mABs, we used an ELISA in which whol borrelias were not dried in the microtiter plate wells. To further evaluate mAbs for surface binding all hybridoma supernatants identified by wet ELISA were examined by unfixed cell

Table 4. Analysis of polyclonal mouse antisera to B311 and B313 cells by ELISA and growth inhibition assay[a]

Polyclonal serum	Mouse No	ELISA[b] B311	ELISA[b] B313	Growth inhibition assay[c] B311	Growth inhibition assay[c] B313
Anti-B311	1	16384	16384	8192	32
	2	16384	16384		
	3	32768	16384		
	4	32768	16384		
Anti-B313	1	4096	16384	<8	4096
	2	4096	16384		
	3	4096	32768		
	4	2048	32768		
Control[d]	1	<4	<4	<8	<8
	2	<4	<4		

[a] Mice were immunized with B311 and B313 whole cell emulsion in CFA and were boosted once with the same immunogen.

[b] Reciprocal ELISA titers from individual mouse sera.

[c] Reciprocal growth inhibitory titers of heat-inactivated (56°C, 30 min) pooled mouse sera.

[d] Control mice were immunized with complete Freund's adjuvant emulsion in PBS.

immunofluorescence assay. Using these assays we identified several mAbs specific for B313 cells.

Six mAbs produced against the Osp-less mutant were selected for further study by Western blot and GIA. We had distinguished two different classes of mAbs, designated A and B, in the screening by unfixed cell IFA. The 3 class A mAbs produced prominent cell blebs and 4+ cell aggregates; the 3 class B mAbs produced 3+ aggregates and did not produce blebs (Figure 1). The morphologic changes observed with class A mAbs were similar to what was observed by Coleman *et al* (25) and us (50) when bactericidal antiborelial antibodies were used. Class A mAbs were associated with a homogeneous patchy pattern of binding to whole cells and little fluorescent staining of the background (see below). In contrast class B mAbs in the wet IFA did not produce staining of single whole cells. Instead it was associated with numerous fluorescent spots in the background (not shown). By GIA class A antibodies were inhibitory at dilutions of hybridoma supernatant of 1:256-2048; class B mAbs inhibited growth only at dilutions of supernatants of 1:16 or lower. Both class A and B mAbs inhibited the growth of B311 at a dilutions of 1:16 or 1:32, but not at higher dilutions. None of the antibodies inhibited the growth of *B. hermsii*. When 1 HU of guinea pig complement was added, it did not increase the inhibitory effect of either class of mAb against B313 cells.

Western blot analysis of mAbs

The two classes were also distinguishable by Western Blot. Class B mAbs did not bind to any protein in the blots, a result that suggested these mAbs were directed against conformational epitopes or non-proteinaceous antigens. In contrast, all class A mAbs were reactive by Western blot and bound to the same low molecular weight protein. The results with two class A mAbs, 15G6 and 7D4, are shown in Figure 2. Both these class A mAbs were IgG2b. We have already described an OspA$^-$OspB$^-$ *B. burgdorferi* mutant of HB19 lineage that expressed a surface protein not detectable in the Osp-bearing wild-typ population (51). Therefore, we first determined whether other lineages of B31 express the protein recognized by 15G6 and 7D4 mAbs. An antibody-reactive protein with an M_r of

13,000 was present in all the B31 cell lineages investigated and in similar amounts. This prot in was designated "p13" and was bound by both mAbs. Identically-sized proteins bound by 15G6 and 7D4 were present in HB19 and Sh2 strains as well (data not shown). Both mAbs also produced minor bands with proteins with with M_r's of 26,000, 32,000, and 44,000 (Fig. 2).

We next determined if mAbs 15G6 or 7D4 mAbs recognized similar or identical proteins in other genomic species of Lyme disease borrelias. The results with 15G6 are shown in Figure 3; the same results were obtained with 7D4. Representatives of *B. afzelii* and *B. garinii* were evaluated at the same time as B311, B313 and *B. hermsii* cells by Western blot. The mAb recognized a protein of slightly higher apparent molecular weight in *B. afzelii* ACAI. Neither 15G6 nor 7D4 recognized any protein in *B. garinii* IP90 or *B. hermsii*.

We also investigated whether p13 was cleaved from intact cells by proteinase K, as has been shown for other *B. burgdorferi* surface proteins (21). No band was observed by Western blot with either anti-p13 kDa mAb after proteinase K digestion of wild-type and Osp-less mutant cells, an indication that p13 is surface-exposed. The result with mAb 15G6 and B313 cells is shown in the right panel of Figure 3.

Immunofluorescence studies of p13

To further assess the topography of p13 in the cell, in particular to determine if p13 is exposed over B313's entire surface, we used fixed and unfixed cells in indirect (IFA) and direct (DFA) immunofluorescence assays. In this series of experiments we used purified 15G6 mAb; for unfixed cell DFA purified 15G6 mAb was conjugated with fluorescein.

In the fixed cell IFA B311 and B313 cells were individually mixed with a suspension of washed rat erythrocytes and coated as a thin smear over the the slides. No fluorescein-labeled spirochetes were seen with either wild-type or mutant cells when cells were exposed to 15G6 mAb. In contrast, anti-flagellin mAb H9724, used as a control, showed uniform fluorescein labelling of fixed to the glass spirochetes, as was described in ref. 12. This suggested that the epitope for the 15G6 mAb was sensitive to the experimental

conditions and treatment required for the sample preparation. Although this epitope was accessible to 15G6 mAb by the Western Blot in the whole-cell lysates, it was not recognized in the dried and fixed borrelias.

We then assessed the binding of fluorescein-labeled antibodies to fixed and unfixed borrelias. B313 cells were examined at 3, 15, 30, 60, and 360 min after addition of the 15G6 conjugate. The cells began to fluoresce within 3 min of addition of the conjugate; the antibody was uniformly distributed over the length of the cell by 30 min (Fig. 4). Cells remained motile for up to 30 min. Cell aggregates and blebs became evident after 15 min and increased in amounts over the 6 hours' observation (Fig. 4). In contrast to B313, very few (<1%) of B311 cells were detectably bound by 15G6 conjugate by DFA with unfixed cells.

The finding that mAb 15G6 had some inhibitory activity against B313 cells, albeit only at a low dilution, suggested that p13 of OsA^+B^+ cells was accessible to some degree to the antibody. We asked whether this putative exposure could be increased in wild-type cells by the additional presence of an anti-Osp antibody in the suspension. To address this question we used either the anti-OspB mAb H6831 or anti-ospA mAb H5332 in combination with the15G6 conjugate. Both antibodies of the combination were added at the same time. The 15G6 conjugate was also used by itself against B311 or B313. Immunofluorescence of cells was examined in 2 h. As expected the conjugate by itself did not bind to B311 cells when used alone (not shown). The conjugate produced aggregation and homogeneous staining of cells of B313 (Fig. 5). In contrast, the binding of the 15G6 conjugate to B311 cells in the presence of the anti-OspA or anti-OspB mAbs was not homogeneous. The results with H6831 and the conjugate are shown in Figure 5. The cells were found in large aggregates with patches of fluorescence dispersed throughout the clump of cells. The experiment showed that simultaneous exposure to mAbs directed against OspA or OspB resulted in exposure of p13 protein by mAb 15G6. Whether this was directly attributable to cell lysis or to alterations of the outer membrane and its proteins remains to be determined.

Functional characterization of anti-p13 mAb

Results of the preceding experiments prompted further investigation of mAb 15G6 at the functional level. For this we used the whole purified IgG molecule and univalent Fab fragments of 15G6 mAb. Two bactericidal antibodies, anti-OspB mAb H6831 and anti-Bh33 mAb H4825 prepared in the same way served as controls (50). All mAbs were tested with B311 and B313 cells by GIA (Table 5). As reported previously, the anti-OspB mAb H6831 was highly effective in killing B311 cells but, as expected, produced no damage on the Osp-less mutant cells. The effect of 15 G6 mAb to the Osp-less cells, however, was marked. The MIC of the whole IgG was 20 ng/ml. Univalent fab fragments inhibited growth at a concentration of 200 ng/ml, the same as was observed with the bactericidal Fab fragment directed against Bh33, H4825 (53). As found previously with hybridoma supernatants, 15G6 in purified form inhibited growth of B311 cells only at 25 µg/ml or above. No growth inhibition with either B313 or B311 was observed with the anti-*B. hermsii* mAb H4825. This experiment proved the functional importance of the newly identified 15G6 mAb to the Osp-less mutant cells and provided evidence that mAbs active as Fab fragments can be produced against other surface proteins besides Osp proteins.

The combination of anti-Osp and anti-p13 mAbs on wild-type cells was further characterized by GIA. Wild-type cells were exposed to two-fold serially diluted purified H6831 (anti-OspB), H5332 (anti-OspA), or, as a control, anti-Bh33 mAb H4825. 15G6 mAb was simultaneously applied at 200 ng/ml, 10X the MIC for B313 and less than 0.01X the MIC for B311. GIA without the addition of mAb15G6 was performed in parallel. The results ar shown in Table 6. There was no effect with mAb H4825, with or without the addition of 15G6 mAb. There was only a two-fold decrease in the MIC of mAb H5332 when mAb 15G6 was added. The effect of the addition of 15G6 to H6831 was more pronounced: without 15G6, its growth inhibitory concentration was 150 ng/ml, whereas with addition of 15G6, intensive cell blebbing occured at concentrations 64-128-fold lower, i.e., 1-2 ng/ml. These results were consistent with observations of the combination by immunofluorescence assay and indicate that anti-Osp and anti-p13 mAbs are synergistic in their activity against

Table 5. Growth inhibition by purified whole IgG and Fab fragments of mAbs 15G6, H6831 and H4825 mAbs

Cells	Minimal growth inhibitory concentration (µg/ml)					
	15G6		H6831		H4825	
	Whole IgG	Fab fragment	Whole IgG	Fab fragment	Whole IgG	Fab fragment
B311	12.5	25	0.15	2	>25	>25
B313	0.02	0.2	>25	>25	>25	>25

Table 6. Growth inhibition by purified whole IgG of mAbs H6831, H5332 and H4825 in combination with mAb 15G6[a].

	Minimal growth inhibitory concentration (ng/ml)		
	H6831	H5332	H4825
Without 15G6	150	600	>25000
With 15G6[a]	1-2	300	>25000

[a] The amount of purified whole IgG of 15G6 mAb was 10X MIC for B313 cells.

B. burgdorferi.

DISCUSSION

An isolate of *B. burgdorferi* lacking OspA, B, C, and D was characterized with respect to biological functions and its surface antigens, in particular a 13 kDa protein. Although we focused on a single mutant of *B. burgdorferi* the results are likely also applicable to other strains of *B. burgdorferi sensu lato* and the other genomic species of Lyme disease agents. Other isolates of Lyme disease borrelias have one or more of the Osp proteins (reviewed in ref. 6). The study showed that the Osp-less mutant differed in several ways from the OspAB-bearing parent with which it was compared with. Although the most prominent structural difference between B311 and B313 was their Osp protein phenotypes, differences in other, less abundant proteins or in non-proteinaceous components may have affected changes in function. The most apparent genetic difference between the $OspA^{+}B^{+}$ B311 and $OspA^{-}B^{-}$ B313 was the presence or absence of the entire 49 kb linear plasmid. Thus, insights into Osp function from this study will need to be further explored by more direct and specific mutagenesis of these genes.

Biological characteristics distinguishing Osp-less and Osp-bearing cells was growth rate and the population density at which stationary phase occurred. Isolate B313 grew more slowly than did B311 and stopped dividing at a lower cell density than did B311. This may be attributable wholly or in part to the greater auto-agglutination displayed by the mutant cells. The triad of self-aggregation, slower growth rate, and lower cell density at stationary phase have also been noted with low-passage, infectious isolates of *B. burgdorferi* (3, 53). Like B313, some low-passage isolates of *B. burgdorferi sensu lato* also have a poor plating efficiency on solid medium (47). The diminished ability of aggregated Osp-less borrelias to move about the broth medium may explain their slower growth under that condition, but why B313 cells could not grow on solid medium when singly dispersed is unknown. Low plating efficiency also is a feature of B314 cells, which lack the 16 kb linear

plasmid as well as the 49 kb plasmid (54). Inasmuch as B314 cells express OspC protein, the lower plating efficiency cannot be attributed to lack of Osp proteins per se.

Curiously, while OspA$^-$B$^-$ cells seem to be inherently more sticky for one another, they were less disposed than OspA$^+$B$^+$ cells to adhere to human endothelial cells. This indicates that the phenomenon of self-aggregation is not equivalent to the association of the borrelias with mammalian cells. Prior studies had revealed functions for OspA in endothelial cell adherence and for OspB in cell penetration (26, 28, 62). The findings of th present study are also consistent with a role for OspA and/or OspB in the association of borrelias with mammalian cells.

We also examined another possible function of Osp proteins, namely resistance to non-immune effects of serum. For a blood-borne pathogen this would seem to be a requirement for successful transmission between hosts and for proliferation within a mammalian host. Much is known about what conferes "serum-resistance" to gram-negative and -positive bacteria; less is known about this aspect of spirochetes. Although borrelias have two membranes sandwiching a peptidoglycan layer, as do gram-negative bacteria, the outer membrane of borrelias appears to be more fluid than that of gram-negative bacteria (8) and lack lipid A-containing glycolipids (61). Thus, it was not likely *a priori* that spirochetes would have a similar mechanism for avoiding the alternative complemente pathway and other non-immune defenses against bacteria. Indeed, the results suggest that OspA and/or OspB protect the cells from complement attack. When OspA, B, C, and D are lacking, the borrelias were more susceptible than OspA$^+$B$^+$ cells to unheated, nonimmune serum and to guinea pig complement.

Whatever protection OspA and OspB appeared to confer to the borrelias in serum did not seem to provide an advantage to cells in skin. In this experiment we used two isolates that are not infectious by the criterion of detectable dissemination to the blood or other tissues. Still, we expected that the Osp-bearing cells would survive for a longer period in the skin than would their Osp-less counterparts. This did not occur in either of th experiments in which this was examined. By 18 hours after inoculation both B311 and

B313 could not be recovered from skin samples placed in culture medium. Infectious isolates persist in the skin for days (14, 47). The limited duration of survival noted in the present study may also be a function of inherent strain differences. A non-infectious isolate of strain HB19 of *B. burgdorferi* survived in the skin for 24 hours by the same culture criterion (49).

Given the indistinguishability of B311 and B313 with respect to skin survival, one might expect that the immune responses to intradermal inoculation of viable borrelias would be comparable. Although the Osp-less mutant lacked two proteins, OspA and OspB, that are immunodominant when syringe inocula of 10^5 or greater are used (13, 29, 30, 46, 56), other antigens, such as flagellin, commonly recognized by antibodies in immune sera were still present. Instead we found that there was little detectable immune response to *B. burgdorferi* by ELISA, GIA, and infectious challenge when B313 was the immunogen. Under the same conditions and with the same dose, mice given B311 had high titers to *B. burgdorferi* by immunoassays and were protected against challenge with strain Sh.2. The experiment's results suggest that OspA and/or OspB not only are immunodominant antigens but also, perhaps through their mitogenic properties (41), immunostimulatory.

This experiment also raised the possibility that there were no antigens on the cell surface in B313 cells. Without Osp proteins, the cell surface of *B. burgdorferi* conceivably could be like *Treponema pallidum's* outer membrane, which is notably inert to the immune system (45). To further assess this we immunized mice with B313 but this time used adjuvant to enhance immune responsiveness. When this was done, the antiserum produced to B313 cells inhibited the growth of homologous cells but only minimally that of B311. The similar ELISA titers for both anti-B311 and anti-B313 sera against homologous and heterologous cells indicated that with the appropriate adjuvant B313 could elicit antibodies to antigens shared with B311. The GIA results showed that there were unique features of th surface of B313 cells. These components were either not expressed by B311 cells or were otherwise cloaked in these cells. The minimal effectiveness of polyclonal anti-B311 sera in inhibiting the growth of B313 cells indicated that antibodies to

OspA and/or OspB conferred growth inhibition.

The remaining antigens of the Osp-less mutant were further investigated with mAbs. The screening procedures were designed to identify antibodies that had the functional activity of growth inhibition. The antibodies selected by this means fell into two classes: one in which the antibodies in broth medium produced large aggregates and prominent membrane blebs and a second in which the antibodies produced smaller aggregates and minimal evidence of lysis. The first antibodies were found to bind to a 13 kDa (p13) protein in Western blots. The second group of antibodies did not bind to any component in blots. For the remainder of this study p13 and mAbs to it were characterized in more detail.

The evidence that the 13 kDa protein was surface-exposed in the Osp-less mutant was the following: (i) agglutination of viable cells by antibody; (ii) growth inhibition by whole immunoglobulin and Fab fragment; (iii) direct immunofluorescent staining of live cells by an antibody conjugate; and (iv) cleavage of antibody's epitope from the cell's surface by *in situ* treatment with protease. p13 was present in all members of the B31 lineage and in approximately equal amounts. The expression of the protein did not vary according the amount of one or another the Osp proteins. A slightly larger protein recognized by the mAb was present in a *B. afzelii* strain. If Ip90, a representative of *B. garinii*, have a homologous protein it does not share the mAbs' epitope.

We considered whether p13 was identical to one of the other low molecular weight *B. burgdorferi* proteins to which antibodies have been developed. Like antibody to p13, antibody to a 10 kDa protein, as reported by Katona *et al.* (36), bound to only a small proportion of Osp-bearing cells in immunofluorescence assays. However, the molecular size of 10 kDa protein did not vary between strains and uniform fluorescein labeling was seen in fixed cell preparation when probed with mAb to 10 kDa protein (36). Furthermore, 15G6 does not bind to the 10 kDa in Western blots (31). Sambri *et al.* reported the presence of a 14 kDa protein of *B. burgdorferi* (55). This was identified with a mAb and by immunofluorescence of live borrelias. In contrast with what was observed by us with mAbs to p13 and by Katona *et al.* with antibody to the 10 kDa protein (31), antibody to the 14 kDa

protein of Sambri *et al.* bound to the majority of cells (48). These differences suggest that p13 is neither the 10 kDa nor 14 kDa proteins of *B. burgdorferi.*

The effect of 15G6 on susceptible borrelias was similar to what was observed with the anti-OspB mAb H6831 (50). Binding to the cells was detectable by direct immunofluorescence by 3 minutes. The staining was homogeneous and was followed by the appearance of membrane blebs and further cell aggregation even with Fab fragments. The concentration of 15G6 mAb at which growth inhibition and cell disruption occurred was 20 ng/ml. This was 10-fold lower to what was observed with H6831 mAb against *B. burgdorferi* and the same as with H4825 against B. hermsii (53).

The failure of mAbs to p13 to inhibit the growth of Osp-bearing cells is consistent with lack of surface exposure of the protein, or at least impairment of the antibody's access to its target. The cloaking or obstruction could be from OspA, OspB, or a complex of the two. It was also possible that p13 was not in the outer membrane at all in B311 cells; in those cells it may have been in the periplasmic space or in the cytoplasmic membrane. Evidence against this latter possibility was (i) cleavage of anti-p13 mAbs epitopes from Osp-bearing cells by *in situ* treatment with proteinase K, (ii) the finding that when mAb 15G6 to p13 was added to antibodies to either OspA or OspB, the growth inhibitory concentration for the anti-Osp antibodies was decreased, substantially with the bactericidal H6831 to OspB. By itself mAb 15G6 had no discernible effect against B311 cells except at high concentrations. The finding suggested that p13 was exposed to mAb 15G6 when antibodies to OspA or OspB gathered together Osp proteins in patches in the fluid outer membrane (12). The immunofluorescence provided visual evidence of this; large membrane blebs of B311 cells treated with anti-OspA or -OspB proteins were bound by conjugated 15G6 mAb. This in vitro synergism between the two antibodies, one directed against an Osp protein and the other against p13, suggests to us that p13 in combination with OspA or OspB could be useful for immunoprophylaxis against Lyme disease.

The results also lead to other questions about the interaction of antibodies and borrelias, in particular those lacking the known Osp proteins. The target or targets for the

second class of mAbs remains to be determined. It is also possible that they also bind to p13 but that their epitopes are sufficiently conformation-dependent that Western blots would be negative. Alternatively, there may be other proteins or other non-proteinaceous components in the outer membrane against which these functional antibodies act.

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BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Phase contrast photomicrograph of aggregation of *B. burgdorferi* B313 by monoclonal antibodies. A (upper) panel, 4+ aggregation (see Methods) by class A antibody. B (lower) panel, 3+ aggregation by class B antibody. Bar, 1.0 µm.

Figure 2. Coomassie blue-stained polyacrylamide gel (CB) and Western blot analysis (WB) of whole-cell lysates of *B. burgdorferi* isolates B31, B311, B312, B313, and B314 with either monoclonal antibody 15G6 and 7D4. The acrylamide concentration was 17%. The molecular size standards (x 1000) were ovalbumin (44), carbonic anhydrase (29), β-lactoglobulin (18), lysozyme (14), and bovine trypsin inhibitor (5.6).

Figure 3. Western blot analysis with antibody 15G6. Left panel, *B. burgdorferi* B311 and B313, *B. afzelii* ACAI, *B. garinii* IP90 and *B. hermsii* Bh33 were probed with the antibody 15G6 mAb. Right panel, B313 cells treated (+) or untreated (-) with proteinase K (PK). The molecular size standards (x 1000) were carbonic anhydrase (29), β–lactoglobulin (18), lysozyme (14), and bovine trypsin inhibitor (5.6).

Figure 4. Binding of flourescein-conjugated monoclonal antibody 15G6 to *B. burgorferi* B313. Left-upper and right-upper panels, direct immunofluorescence of unfixed cells in suspension for 3 min (left) and 15 min (right). Lower panel, phase contrast photomicrograph of aggregates and membrane blebs (arrow head). Bar, 10 µm.

Figure 5. Phase contrast photomicrographs (left) and direct immunofluorescence (right) of unfixed *B. burgdorferi* in suspension. A (upper) panels , B313 cells with fluorescein-conjugated antibody 15G6. B (lower) panels, B311 cells with unconjugated antibody H6831 and conjugated antibody 15G6. 15G6 alone did not bind to B311 cells (not shown). Bar, 2.0 µm. Figure 5 has four panels, two in A and two in B; and no further panels or drawing elements.

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The above references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are all specifically incorporated herein by reference.